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Direct interferon-γ-mediated protection caused by a recombinant coxsackievirus B3

Andreas Henke,* Roland Zell, Ulrike Martin, and Axel Stelzner

Institute of Virology and Antiviral Therapy, Medical Center, Friedrich Schiller University, Winzerlaer Strasse 10, D-07745 Jena, Germany

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Abstract

Coxsackievirus B3 (CVB3) is one of the most important causes of viral myocarditis. Cytokines are involved in the control of CVB3 replication and pathogenesis. Local expression of specific cytokines by recombinant CVB3 confers prevention of virus-caused myocarditis. Expression of IFN-γ by CVB3(IFN-γ) protected BALB/c and C57BL/6 mice when the lethal infection with the highly pathogenic CVB3H3 variant was given directly after or prior to CVB3(IFN-γ) inoculation by decreasing the viral load and spread as well as tissue destruction. This direct effect was not restricted to the homologous virus. In vitro, cocultivation of CVB3(IFN-γ)-infected cells induced a reduction of CVB3H3 replication and virus-induced cytopathogenicity.

Keywords: Recombinant coxsackievirus; Myocarditis; Interferon-γ; Protection; Therapy

Introduction

Cardiovascular disease is one of the major causes of human death and has been linked to many different risk factors. Coxsackievirus B3 (CVB3), a member of the Picornavirus family, is an important human pathogen that has been associated with serious diseases including acute and chronic myocarditis and pancreatitis (Baboonian et al., 1997; Clements et al., 1995). Extensive analysis indicates that coxsackieviruses, together with other enteroviruses of the picornavirus family, are involved in up to 50% of acute myocarditis and in approximately 25% of cases characterized by symptoms of dilated cardiomyopathy (DCM) in humans. Each year, around 100,000 new cases of DCM are diagnosed in the U.S.A. (Bowles et al., 1986). The presence of CVB3 in heart tissue of patients with acute or chronic myocarditis has been demonstrated (Kandolf et al., 1987). However, the pathogenesis of coxsackievirus-induced human heart disease is only partly characterized so far. Therefore, coxsackievirus-caused myocarditis has been studied extensively in different murine models (Chow et al., 1992; Henke et al., 1995; Huber and Pfaeffle, 1994; Leipner et al., 1999; Mena et al., 1999; Wolfgram et al., 1986), demonstrating that the outcome of this viral infection is determined by complex interactions among several variables of the virus and the host (Chow et al., 1991; Huber, 1997). Despite the well-characterized molecular structure of coxsackieviruses (Natarajan and Johnson, 1998) and the successful use of common (Fohlman et al., 1993; See and Tilles, 1997) or novel immunization procedures (Henke et al., 1998, 2001b) in animal models, no virus-specific preventive procedures against CVB3-caused myocarditis are in clinical use today.

Experimental data indicate that cytokines are involved in controlling CVB3 replication. For example, IFN-γ especially was shown to be highly protective against coxsackievirus-induced disease because IFN-γ knockout mice were completely susceptible to this infection (Horwitz et al., 1999). Otherwise, an organ-specific transgenic overexpression of IFN-γ significantly reduced CVB3 and CVB4 replication in murine pancreas tissue (Horwitz et al., 1999, 2000). The simultaneous expression of those immunoregulatory proteins from within the viral genome and starting immediately with the viral replication should activate immune responses much faster than during a normal course of infection. Recently it has been demonstrated that a CVB3

* Corresponding author. Fax: +49-3641-657202.
E-mail address: i6hean@rz.uni-jena.de (A. Henke).

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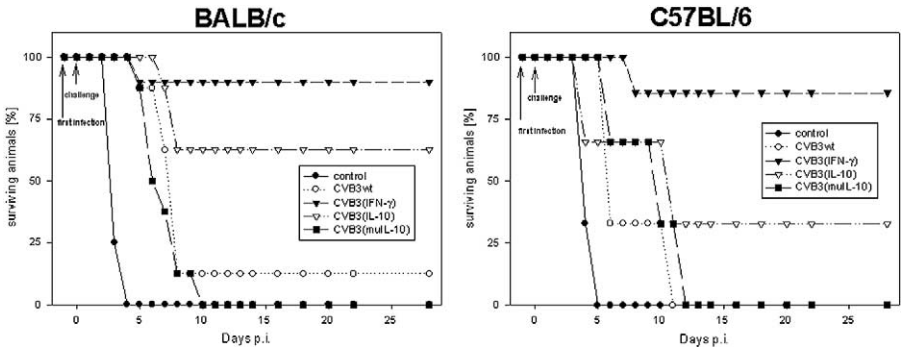

Fig. 1. Preventive effect of cytokine expression by recombinant CVB3 variants. BALB/c and C57BL/6 mice were inoculated with CVB3wt, CVB3(IFN-γ), CVB3(IL-10), or CVB3(muIL-10). Control mice remained noninfected. One day later, all mice were challenged with 100 LD_{50} doses of CVB3H3 i.p. The percentage of animals surviving is shown over a period of 28 days. The results presented summarize data from three independent experiments, using at least three to four mice in each group.

vector can produce an intact bioactive interleukin (interleukin-4) under in vitro and in vivo conditions (Chapman et al., 2000). Furthermore, the simultaneous expression of the immunoregulatory cytokines IL-10 and IFN-γ—released by recombinant CVB3 variants (CVB3rec)—influenced the normal pattern of immune pathways and caused an intense protective reaction against subsequent viral infections (Henke et al., 2001a). Four weeks after the initial infection with CVB3rec, challenge experiments with the lethal variant CVB3H3 revealed almost complete protection against CVB3 infections using the genetically different BALB/c or C57BL/6 mice, depending on the virus-caused production of functional cytokines by CVB3rec variants regardless whether the T_H1- or T_H2-specific cytokines were expressed.

In addition to this indirect protective effect, the direct cytokine-mediated influence on the replication of a highly pathogenic CVB3 strain (CVB3H3) was analyzed under in vivo and in vitro conditions. This study focused on (i) challenge experiments by which the lethal infection was given directly after or prior to the administration of CVB3rec variants, (ii) the influence of this cytokine expression on the replication and cell destruction caused by a homologous or heterologous coxsackievirus, and (iii) the characterization of the putative protective mechanism via the induction of the inducible nitric oxide synthase (iNOS).

Results

Direct protective activity in vivo

A high level of virus replication, spread to susceptible organs, and extensive myocardial tissue damage are responsible for disease and death in this murine model of CVB3-induced myocarditis. After intraperitoneal (i.p.) infection, viral replication starts in exocrine pancreas tissue at first. Thereafter, infectious virus spreads via the blood circulation to different susceptible organs, e.g., spleen and heart, causing cadiomyocyte destruction and death. One strategy to prevent this damaging event is the application of cytokine-expressing CVB3 variants 4 weeks prior challenge. As it was shown before (Henke et al., 2001a), the replication of all recombinant CVB3 variants was almost equal with regard to the localization (pancreas only) and the amount of infectious viruses, indicating an attenuated phenotype. In contrast, CVB3wt (the parental strain of the CVB3rec variants) induced destruction of the exocrine pancreatic tissue, and virus spread to several organs, including the heart. In addition, only BALB/c mice—which were inoculated with the CVB3(IFN-γ) variant—revealed increased serum IFN-γ concentrations (65–125 pg/ml) 1 day postinfection (p.i.), whereas in sera of all other infected mice basal IFN-γ levels were present (15–70 pg/ml).

To analyze the direct preventive effect of the cytokine expression by recombinant CVB3 variants on a lethal infection with the highly pathogenic strain CVB3H3, BALB/c as well as C57BL/6 mice were intraperitoneally inoculated with CVB3rec variants prior to or after the CVB3H3 challenge. The CVB3(muIL-10) construct—which expresses only a biologically inactive form of IL-10 (Henke et al., 2001a)—was used to distinguish between the attenuated phenotype of the CVB3rec variants and the real cytokine effect under in vivo conditions. Mice of certain control groups either received CVB3wt or remained noninfection. Different mouse strains were used to demonstrate the efficacy of the treatment independently of the genetic background of mice. One day later, these mice were challenged with 100 LD_{50} doses (BALB/c: 16,440 PFU; C57BL/6: 52,000 PFU) of the lethal variant CVB3H3. After the challenge, the number of surviving animals was monitored up to 28 days p.i. No excess mortality was noted beyond this time. As shown in Fig. 1, 87.5% of the CVB3(IFN-γ)-inoculated BALB/c mice survived this lethal challenge. Preinjection with CVB3(IL-10) or CVB3wt was much less protective, because only 62.5% or 12.5% survived the CVB3H3 infection. No control mice and no mice of the CVB3(muIL-10)-treated group were protected. The statistical analysis demonstrates significant differences between the CVB3(IFN-γ)-

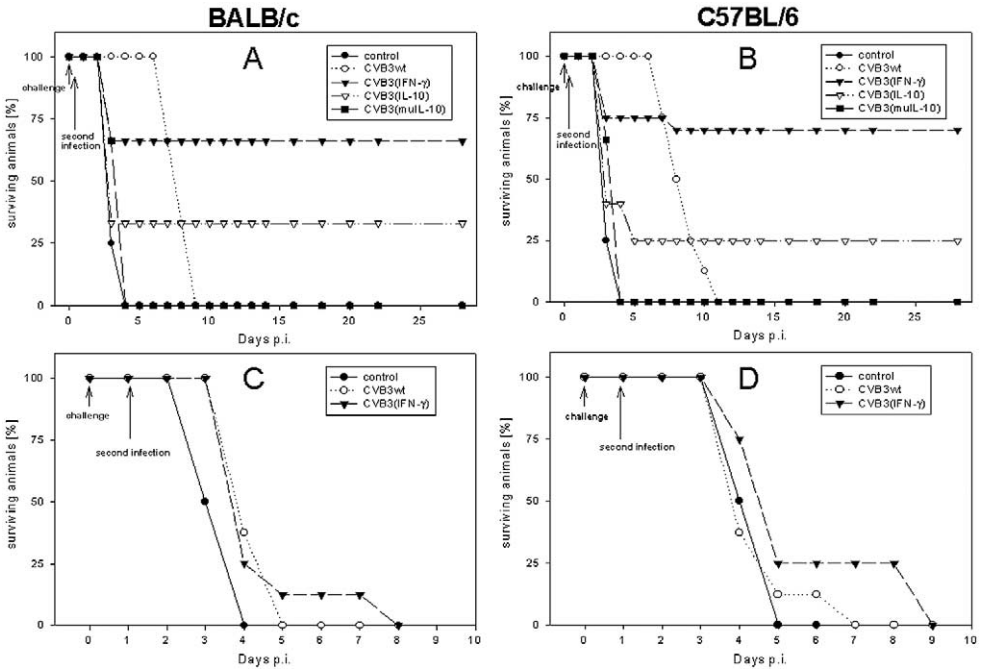

Fig. 2. Therapeutic effect of cytokine expression by recombinant CVB3 variants. BALB/c (A and C) and C57BL/6 (B and D) mice were infected with five LD_{50} doses of CVB3H3 i.p. Six hours (A and B) or 24 h (C and D) later, mice were inoculated with CVB3wt, CVB3(IFN-γ), CVB3(IL-10), or CVB3(muIL-10) (A and B) or only with CVB3wt or CVB3(IFN-γ) (C and D). Control mice did not receive a second infection. The percentage of animals surviving is shown over a period of 28 days. The results presented summarize data from three independent experiments, using at least three to four mice in each group.

and the CVB3(IL-10)-treated mice ($P < 0.0014$). With C57BL/6 mice, a similar result was obtained. After CVB3(IFN-γ) inoculation, 85.7% survived, whereas only 33.3% of the CVB3(IL-10) were protected. As well, the statistical analysis demonstrates significant differences between the CVB3(IFN-γ)- and the CVB3(IL-10)-treated group ($P < 0.00003$). Mice of all other groups did not survive the 28-day observation period.

Next, the inoculation schedule was changed in a way that at first BALB/c or C57BL/6 mice were i.p. infected with five LD_{50} doses (BALB/c: 822 PFU; C57BL/6: 2600 PFU) of CVB3H3. Six hours later, these mice were inoculated with 10^6 PFU of the CVB3rec variants. Control groups either received CVB3wt or remained noninfected. After that, the number of surviving mice were monitored up to 4 weeks p.i. No excess mortality was noted beyond this time. As shown in Figs. 2A and B, up to 66% of BALB/c mice and 70% of C57BL/6 mice—which were treated with the IFN-γ-expressing CVB3 variant—were protected against the lethal challenge, indicating a IFN-γ-specific effect. The treatment with CVB3(IL-10) caused only 30% (BALB/c) and 25% (C57BL/6) protection, whereas mice of both strains, which received either CVB3wt or CVB3(muIL-10), were not protected at all. The comparison of the surviving rate between CVB3(IFN-γ)- and CVB3(IL-10)-inoculated BALB/c or C57BL/6 mice revealed statistically increased values for the CVB3(IFN-γ)-inoculated group: BALB/c mice: $P < 0.0009$; C57BL/6 mice: $P < 0.0004$. These challenge experiments clearly demonstrated that IFN-γ directly reduce mortality during an ongoing acute CVB3 infection in mice. The same experiments—carried out 24 h after the lethal infection and with the efficient CVB3(IFN-γ) variant—revealed no protection as is demonstrated in Figs. 2C and D. However, the IFN-γ expression by CVB3(IFN-γ) delayed the onset of this lethal disease in animals of both mouse strains, indicating the antiviral interferon activity.

To confirm that the IFN-γ-caused protection against CVB3H3 was not limited to the homologous virus, the heterologous coxsackievirus B4 (CVB4) was applied. The CVB4 strain—used in this experimental setup—replicates to high titers in the pancreas of BALB/c mice causing an extensive destruction of the exocrine tissue but no death after i.p. inoculation. An example of this observation is demonstrated in Fig. 3A. However, when mice were inoculated with CVB3(IFN-γ) 24 h prior CVB4 infection, no pancreas tissue destruction was detectable up to 4 weeks after this second infection (Fig. 3B). In contrast, this tissue protection was unobservable when mice were inoculated with CVB3wt, CVB3(IL-10), or CVB3(muIL-10) 1 day prior CVB4 infection (Figs. 3C, D, and E).

Characterization of the direct protective activity in vivo

Further experiments focused on the IFN-γ-caused influence on CVB3H3 replication. Therefore, BALB/c mice were i.p. inoculated with CVB3wt or CVB3(IFN-γ), respec-

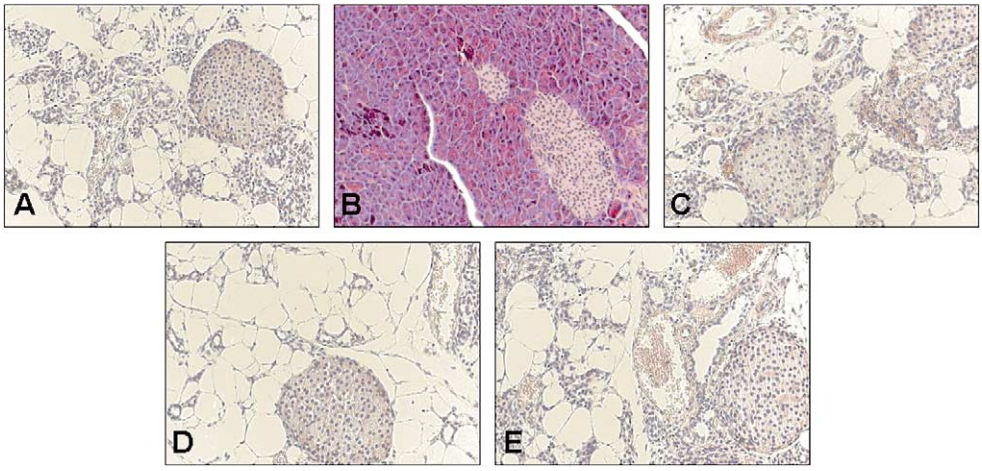

Fig. 3. Infection with CVB4. Paraffin sections of pancreas tissue obtained from BALB/c mice were stained with hematoxylin and eosin (magnification, ×200). Mice were infected with CVB4 (A) or inoculated with CVB3(IFN-γ) (B), CVB3wt (C), CVB3(IL-10) (E), or CVB(muIL-10) (F) 24 h prior CVB4 infection. All samples were taken 4 weeks after the last infection. Only the exocrine pancreas tissue of CVB3(IFN-γ)-inoculated CVB4-infected mice remained undamaged (B).

tively. One group of mice remained noninfected. One day later, all mice were infected with 100 LD_{50} doses of the lethal CVB3H3 strain. Again, 1 day later, all mice were sacrificed and the amount of infectious virus was analyzed in different tissues, such as pancreas, spleen, and heart, as well as in sera. As shown in Fig. 4, the virus load in control mice—which received only the lethal challenge—and in mice CVB3wt-inoculated before the challenge infection—was high in pancreatic tissue and well detectable in heart, spleen, and serum. In contrast, the virus concentration in pancreas tissue of CVB3(IFN-γ)-inoculated BALB/c mice was significantly reduced ($P < 0.002$) and no virus was detectable in spleen, heart, or serum. Also by RT-PCR technique no viral RNA was found in these tissue samples (data not shown). As expected, this effect was based on the expression of IFN-γ by CVB3(IFN-γ), because in the CVB3wt-inoculated group, a normal virus replication occurred. Using C57BL/6 mice, basically the same results were obtained during these challenge experiments (Fig. 4). Again, due to the IFN-γ expression by CVB3(IFN-γ), the virus load was significantly reduced in the pancreas ($P < 0.002$) and not detectable in spleen, heart, and serum. Later on during these challenge experiments, in CVB3H3- or CVB3wt/CVB3H3-infected BALB/c and C57BL/6 mice, the virus load in the heart increased causing myocardial damage and death. In contrast, in CVB3(IFN-γ)-treated mice, the CVB3H3 spread to other organs was completely inhibited. Taken together, these results indicate a direct virus-inhibitory effect of IFN-γ after expression by the recombinant virus variant CVB3(IFN-γ), which prevents CVB3-induced disease.

To analyze the fate of the challenge virus after infection, male BALB/c mice were inoculated with either CVB3wt or CVB3(IFN-γ), or remained noninfected (control). One day later, mice were challenged with 100 LD_{50} doses of CVB3H3 and 1 day after this challenge, RNA was isolated from pancreas and heart tissue. By RT-PCR procedure, the coding sequence for the structural protein VP1 was amplified as shown in Fig. 5. Due to the fact that an unique *Eco*RI restriction site is located only in the VP1 sequence of CVB3H3, a differentiation between the viruses (CVB3H3 versus CVB3wt and CVB3H3 versus CVB3(IFN-γ) was possible. In tissue of individual BALB/c mice—which were only inoculated with the challenge virus—the obtained VP1 PCR fragment is cleaved with *Eco*RI into two distinct fragments. In contrast, in tissue of BALB/c mice—which were inoculated with CVB3wt or CVB3(IFN-γ) prior the CVB3H3 challenge—the VP1 PCR product was not cleavable with *Eco*RI, indicating that at this time the challenge virus was not detectable by this method. This result was confirmed by sequence analysis. However, later during the challenge experiments, CVB3H3 overcame this inhibition and started to replicate in pancreas and heart tissue of mice previously infected with CVB3wt, causing tissue destruction and death. In contrast, in heart tissue of CVB3(IFN-γ)/CVB3H3-infected animals no infectious virus and no viral RNA was detectable during the challenge period, confirming the data presented in Fig. 4.

During murine coxsackievirus infections the expression of iNOS is well documented in several studies (Lowenstein et al., 1996; Zaragoza et al., 1998; Glück et al., 2000), indicating that iNOS is directly induced in certain CVB3-infected tissue. IFN-γ is capable of activating iNOS (Karupiah et al., 1993), which may lead to the production of nitrite oxide (NO) and thereby may prevent viral replication by inhibiting the viral protease 2A (Badorff et al., 2000). To analyze that the IFN-γ expression by CVB3(IFN-γ) activates the iNOS transcription early during infection, equal RNA amounts from pancreas tissue of individual mice—which were either inoculated with CVB3H3 alone or with CVB3wt and 24 h later with CVB3H3 or with CVB3(IFN-γ) and 24 h later with CVB3H3—were isolated,

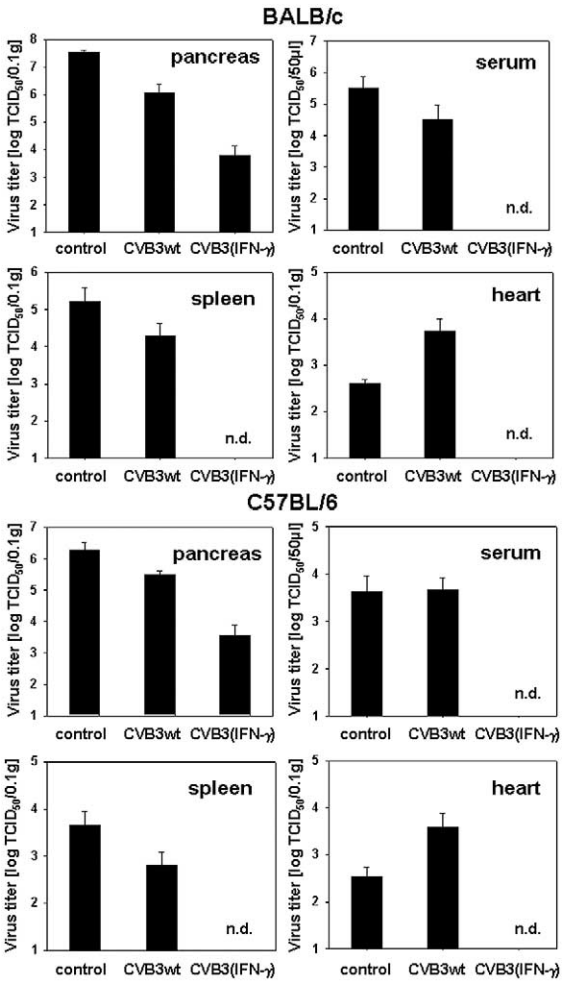

Fig. 4. Quantification of infectious virus. The amount of infectious virus particles in pancreas, serum, spleen, and heart of BALB/c as well as C57BL/6 mice 1 day after a lethal challenge with 100 LD_{50} doses of CVB3H3 is characterized by $TCID_{50}$ assays. One day prior to the challenge, mice were inoculated with either CVB3wt or CVB3(IFN-γ), or remained noninfected (control). Experimental groups consisted of five mice, and experiments were repeated three times. The mean ± standard deviation is shown (n.d.: not detectable).

DNase-treated, and reverse transcribed into cDNA. Detection of β-actin mRNA confirmed that all RNA preparations were equally well performed. Using these samples, an iNOS-specific, nonquantitative PCR was performed, as is demonstrated in Fig. 6. In two of three CVB3(IFN-γ)-inoculated mice, the transcription of iNOS was substantially increased in pancreas tissue at this time. However, in one of three CVB3wt-infected mice, the transcription of iNOS was slightly increased over the background level as well. This experiment was repeated three times with three mice in each group. In all three experiments two of three (66%) CVB3(IFN-γ)-infected mice revealed evaluated iNOS mRNA levels, whereas in one of three experiments the iNOS mRNA level in tissue of CVB3wt-infected mice was only slightly increased in one of three mice. Despite individual differences, these results may support the observation that iNOS and therefore possibly NO could be involved in the prevention of CVB3H3 replication.

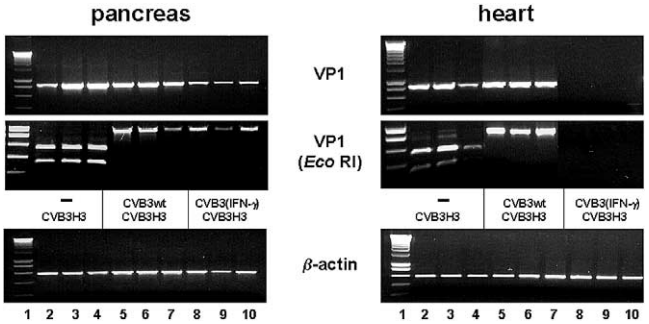

Fig. 5. Differentiation of inoculated virus. Male BALB/c mice were inoculated with either CVB3wt or CVB3(IFN-γ), or remained noninfected (control). One day later, mice were challenged with 100 LD_{50} doses of CVB3H3. One day after this challenge, RNA was isolated from the pancreas and heart tissue of three individual mice. By RT-PCR procedure, the coding sequences of the structural protein VP1 and of β-actin (as an internal control) were amplified. Because a unique *EcoRI* restriction site is located only in the VP1 sequence of CVB3H3, a differentiation between the viruses (CVB3H3 versus CVB3wt and CVB3H3 versus CVB3(IFN-γ)) is possible (1: DNA marker; 2–4: CVB3H3 only; 5–7; CVB3wt and CVB3H3; 8–10: CVB3(IFN-γ) and CVB3H3). Incubation of the CVB3H3 VP1 RT-PCR product (851 bp) with *EcoRI* caused the formation of two fragments (538 and 313 bp), whereas the VP1 RT-PCR product of CVB3wt and CVB3(IFN-γ) remained noncleaved.

Furthermore, the direct antiviral activity of IFN-γ, expressed by the recombinant CVB3(IFN-γ) variant, was also characterized under in vitro coculture conditions. For these experiments, CVB3H3-infected GMK cell monolayers were cocultivated with GMK cells, which were either infected

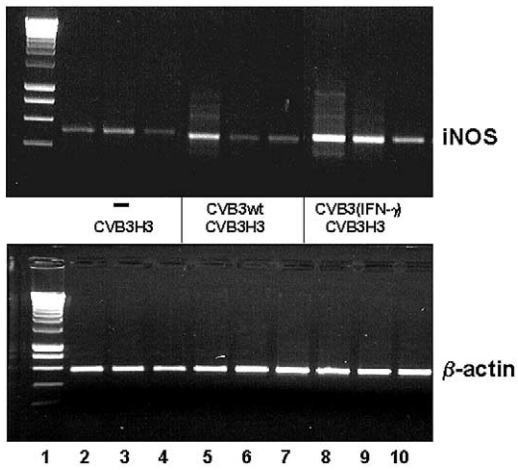

Fig. 6. Detection of iNOS expression. Male BALB/c mice were inoculated with either CVB3wt or CVB3(IFN-γ), or remained noninfected (control). One day later, mice were challenged with 100 LD_{50} doses of CVB3H3. One day after this challenge, RNA was isolated from the pancreas tissue of three individual mice. By RT-PCR procedure, the coding sequences for murine iNOS and for β-actin (as an internal control) were amplified (1: DNA marker; 2–4: CVB3H3 only; 5–7: CVB3wt and CVB3H3; 8–10: CVB3(IFN-γ) and CVB3H3). The results presented summarize data from three independent experiments, using at least three mice in each group.

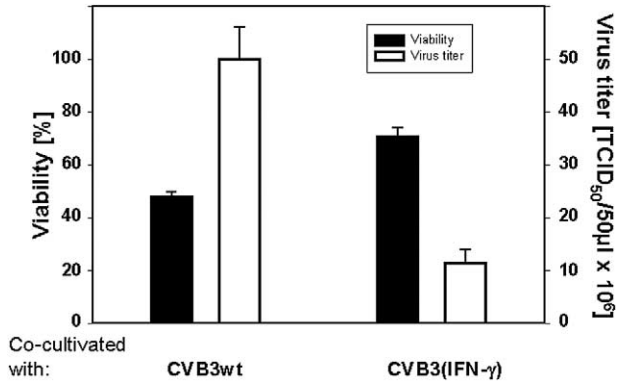

Fig. 7. Inhibition of CVB3wt replication by cocultivation with CVB3(IFN-γ). CVBH3-infected GMK cell monolayers in 24-well cell-culture plates were cocultivated with GMK cells, which were infected with either CVB3wt or CVB3(IFN-γ). Cell monolayers were infected with a m.o.i. of 3. After 1 day of incubation, the viability (in % of noninfected control cultures) of these CVB3H3-infected cells and the amount of infectious virus particles in the supernatants of these cultures were determined.

with CVB3wt or CVB3(IFN-γ). Because of the 20-nm pores in the membrane, only proteins—such as IFN-γ—but not whole virus particles were able to move between both cell cultures during the incubation time. As was expected, the expression of IFN-γ by CVB3(IFN-γ) caused a significant reduction of viral replication ($P < 0.00007$) and virus-induced cytopathogenicity ($P < 0.0027$) in CVB3H3-infected cell cultures in comparison to CVB3H3-infected cells, which were cocultivated with CVB3wt-infected cells 24 h p.i. (Fig. 7). Incubations with different concentrations of recombinant human IFN-γ reduced the viral replication as well, whereas the addition of murine IFN-γ antibodies blocked the IFN-γ-mediated antiviral effect (data not shown).

Discussion

According to the World Health Organization, 1.5% of enteroviral infection and 3.2% of coxsackievirus B infections lead to cardiovascular symptoms (Grist et al., 1978). So far, there is no preventive or therapeutically applicable procedure in clinical use. After experimental CVB3 infection of mice, replicating viruses can be detected during the acute stage of infection in several organs but especially in heart and pancreas tissue. Virus-caused tissue damage and immune activation via cytokine expression are involved in CVB3-caused pathology (Knowlton and Badorff, 1999). As was demonstrated before (Henke et al., 2001a), the simultaneous expression of cytokines such as IFN-γ or IL-10 from within the viral genome and starting immediately with the viral replication activate protective immune responses against subsequent infections (Henke et al., 2001a). Two sites have been used successfully to insert larger foreign nucleotide sequences into picornaviral genomes: the start of the translation (Andino et al., 1994) and the junction of the viral capsid protein 1D and the viral protease 2Apro (Tang et al., 1997). Until today, several studies have described coxsackieviral vectors to express foreign sequences (Chapman et al., 2000; Feuer et al., 2002; Henke et al., 2001a; Hofling et al., 2000; Reimann et al., 1991; Slifka et al., 2001).

IFN-γ is a cytokine produced by NK cells, $CD4^+$ Th1 cells, and a subset of $CD8^+$ cells. This cytokine is considered to be an important defense mechanism during the immune response against intracellular bacteria, certain parasites, and viruses (Billiau, 1996). IFN-γ exerts two major effects: directly inhibiting the replication of some microbes and stimulating the cellular immune response. The direct IFN-γ-mediated effect is based on the induction of certain cellular products that interfere with viral replication (Karupiah et al., 1993) or promote the induction of apoptosis of infected cells (Henry et al., 1993). In CVB3-infected carrier state cultures of human myocardial fibroblast, recombinant IFN-β and IFN-γ possessed a higher antiviral activity than IFN-α (Heim et al., 1996). The indirect IFN-γ effect on the generation and function of specific immune effectors is very complex, including up-regulation of antigen expression (Geginat et al., 1997) as well as modulation of priming (Freedman et al., 1991), recruitment (Taub et al., 1993), and death of activated T cells (Liu and Janeway, 1990). IFN-γ also exerts stimulatory effects on certain functions of macrophages and NK cells (Dalton et al., 1993).

It was recently published that a tissue-specific IFN-γ overexpression in pancreas of IFN-γ-transgenic mice had an inhibitory influence on the replication of CVB3 and CVB4 (Horwitz et al., 1999, 2000). In contrast, histopathological changes were much more distinct in CVB3-infected IFN-γ knockout mice combined with a higher lethality (Horwitz et al., 2000). This observation is consistent with our data demonstrating that the simultaneous and direct local presence of IFN-γ in CVB3-infected tissue dramatically reduces viral replication and prevents death, even when the lethal infection has already started. This direct effect was time-dependent but independent from the genetic background of the infected host or the applied virus (see Figs. 1–5). Therefore, the observed protection of CVB3(IFN-γ)-inoculated mice after the lethal challenge could be based on the circumvention of virus replication. This obvious prevention of CVB3H3 replication can only be explained by a IFN-γ-based activity, because at this early time of infection, no virus-specific antibody response was detectable (data not shown). The result was confirmed by using CVB4-infected animals (Fig. 3). Despite the fact that CVB3 and CVB4 are closely related viruses, virus-specific neutralizing antibodies clearly distinguish between both viruses. Prior to CVB4 infection, only the pancreas tissue of CVB3(IFN-γ)-inoculated mice remained unharmed. Therefore, it is more likely that an IFN-γ-mediated interference between both viruses occurred as it was first described many years ago for the

interferon-caused inhibition of viral replication (Lindenmann, 1982).

The characterization of the nature of the replicating viruses in CVB3wt- or CVB3(IFN-γ)-inoculated mice 1 day after CVB3H3 infection demonstrated, that—despite high viral titer—the challenge virus CVB3H3 was not detectable in pancreas and heart tissue of these mice at this time (Fig. 5). However, most of the mice—which were inoculated with CVB3wt, CVB3(IL-10), or CVB3(muIL-10)—did not survive the lethal CVB3H3 challenge, indicating that CVB3H3 was able to overcome this interference during the challenge period. In contrast, most of the CVB3(IFN-γ)-inoculated mice survived the challenge, which demonstrates the direct antiviral activity of IFN-γ against CVB3. IL-10 expression by CVB3(IL-10) induced some protection against death during challenge experiments (Figs. 1, 2A and B). This effect is based on the bioactive form of IL-10, because inoculations with CVB3(muIL-10) were not protective at all. In addition, the expressed IL-10 revealed no direct tissue-protective antiviral properties, because CVB3(IL-10) was unable to protect exocrine pancreas tissue after CVB4 infection (Fig. 3E) or CVB3H3 challenge (data not shown). In contrast, the protective IFN-γ effect was not virus-specific. BALB/c mice, which received a CVB3(IFN-γ) inoculation 24 h prior a CVB4 infection, were completely protected against virus-induced pancreas destruction (Fig. 3B). Therefore, the IL-10-induced protection to prevent death during CVB3H3 challenge seems to be indirect via the induction of other immune factors. At present, an explanation of this observation can only be speculative. However, it could be demonstrated that immunization with a live-attenuated varicella-zoster virus vaccine induced IL-10 production in human volunteers and was the prerequisite for a primary immune response (Jenkins et al., 1998).

CVB3wt inoculation—given 6 h prior CVB3H3 challenge—significantly delays death (Figs. 2A and B). An explanation for this observation could be that five LD_{50} doses of CVB3H3 equals only 822 PFU (BALB/c) and 2600 PFU (C57BL/6), respectively. After 6 h the first round of CVB3H3 replication was not completed. Six hours after the i.p. administration of this relatively small virus amount, 10^6 PFU CVB3wt or the CVB3rec variants were i.p. inoculated as well. Under in vitro and in vivo conditions the replication capacity of CVB3wt and CVB3H3 is equal, but CVB3wt is by far less lethal than CVB3H3 in mice. Because of the imbalance between the inoculated virus amounts, the initial CVB3H3 replication was affected which could be responsible for the delay of death in these experimental groups. This effect was not detectable when the time between both inoculations was extended to 24 h (Figs. 2C and D). Due to the insertion of additional gene sequences, all recombinant CVB3 variants revealed decreased replication capacities (Henke et al., 2001a). Therefore, using mice, which received 10^6 PFU of CVB3rec variants 6 h after the challenge, this inhibitory impact was not observable.

One important antiviral effect of IFN-γ consists of induction of the iNOS (Harris et al., 1995), which catalyzes the formation of NO from L-arginine. This bioreactive molecule possesses regulative and cytoprotective as well as antiviral activities. During cell-culture experiments (Zaragoza et al., 1997) and murine coxsackievirus infections (Lowenstein et al., 1996; Zaragoza et al., 1998; Glück et al., 2000), the expression of iNOS was well documented in several studies indicating that (i) iNOS is directly induced in certain virus-infected tissue (Mikami et al., 1996); (ii) iNOS protects mice against viral replication (Zaragoza et al., 1999; Flodstrom et al., 2001); but (iii) iNOS may also cause a NO-dependent cytotoxic effect (Bevan et al., 2001). However, an direct inhibitory effect of NO on proteases 2A and 3C of CVB3 has been published recently (Badorff et al., 2002; Saura et al., 1999). Furthermore, iNOS knockout mice revealed an increased mortality rate following coxsackieviral infection in comparison to normal mice (Flodstrom et al., 2001). Our data emphasize these previous results. As is shown in Fig. 6, increased transcriptional activity of iNOS was detectable in two of three individual mice, which received CVB3(IFN-γ) prior to the lethal challenge. In pancreas tissue of one of three CVB3wt-infected mice marginally increased activity was present as well in comparison to the control group. Despite these individual differences, this iNOS induction followed by NO synthesis could be involved in the direct protective activity of IFN-γ produced by CVB3(IFN-γ). Further experiments are focused on that observation. However, in our coculture system (Fig. 7), the virus-replication in the CVB3H3-infected compartment was not completely inhibited by the IFN-γ release, indicating that under in vivo conditions additional antiviral activities were induced.

Materials and methods

Mice

Male inbred BALB/c (H-2^{dd}) and C57BL/6 (H-2^{bb}) mice—7–9 weeks of age—were used in this study. Experimental groups consisted of a minimum of three to four mice, and experiments were repeated usually three or four times. Animal experiments complied with all federal permissions, guidelines, and institutional policies.

Viruses and cell lines

The CVB3 variants used were cDNA-generated viruses obtained after transfection of GMK cells with the plasmids pCVB3wt (Lindberg et al., 1992), pCVB3M2(IFN-γ) (Henke et al., 2001a), pCVB3M2(IL-10) (Henke et al., 2001a), pCVB3(muIL-10) (Henke et al., 2001a), or pBK-CMV/CVB3H3 (Knowlton et al., 1996). The CVB4 strain JVB was obtained from its own common virus stock. All viruses were propagated in GMK cells and quantified by

standard plaque formation assay on GMK cell monolayers as described previously (Henke et al., 1995).

Infection protocol

Mice were inoculated by i.p. injection of 0.2 ml saline containing the stated amount of virus and were monitored daily for morbidity and mortality up to 4 weeks p.i.

Challenge experiments

According to the stated time of the initial infection with CVB3wt or recombinant CVB3 variants, all surviving mice were challenged with 5 or 100 LD_{50} doses of the lethal CVB3H3 variant by i.p. infection. Age-matched control mice, which were not infected during the first set of experiments, were used to verify the outcome of the lethal challenge. Mice were monitored daily for morbidity and mortality up to 4 weeks p.i. The statistical comparisons were carried out with Microsoft Excel by using Student's *t* test.

Virus titer in organs

At different days p.i., organs were aseptically obtained, washed with sterile saline, and homogenized with cell-culture medium (DMEM) containing 50 U of penicillin and 50 mM streptomycin per milliliter. Cell debris was removed by centrifugation and supernatants were subjected to sequential 10-fold dilutions in DMEM. The virus titer was determined by tissue culture infectious dose 50% ($TCID_{50}$) assays. The statistical comparisons were carried out with Microsoft Excel by using Student's *t* test.

Preparation and staining of routine histology

Aseptically removed pancreas and heart tissue were fixed with 4% formaline and mounted in paraffin, and 6-μm sections were cut and stained with hematoxylin-eosin or with Sirius red.

RT-PCR

RNA was isolated from pancreas of infected and noninfected BALB/c mice according to the acid guanidinium thiocyanate phenol chloroform method (Chomczynski and Sacchi, 1987) and described in detail in Henke et al. (2000). The detection of viral genomes was performed by RT-PCR using a primer pair which corresponds to the position 2453–2474 and 3283–3304 of the original cDNA giving a 851-bp PCR product. The *Eco*RI restriction site is located at position 2766 in the cDNA sequence of CVB3H3. For the murine iNOS detection, a commercially available primer pair (Clontech Laboratory) was used, amplifying a 496-bp PCR product.

Coculture experiments

GMK cell monolayers were prepared in 24-well cell-culture plates and 0.02-μm anapore membrane tissue culture inserts (Nalge Nunc Internation). These inserts were placed on the top of monolayers in 24-well plates. GMK cells in 24-well plates were infected with a multiplicity of infection (m.o.i.) of 3 or remained noninfected (control). Cell cultures in the insert were either infected with 3 m.o.i. CVB3wt or CVB3(IFN-γ) or remained noninfected. One day later, the amount of infectious virus particles in the 24-well compartment was analyzed by $TCID_{50}$ assays. The viability of the remaining GMK cells in the 24-well compartment was characterized by crystal violet staining. Briefly, culture supernatants were removed; the monolayers were washed twice with PBS, and crystal violet (0.2% in 20% methanol, 3.5% formaline) was added for 2 h. Thereafter, plates were washed and dried, and the dye was extracted and dissolved for 30 min in 30.5 mM sodium citrate and 0.0195 N HCl in 47.5% ethanol at pH 4.2. The optical density (OD) of the extracted dye was measured at 490 nm. Viability is expressed as an index in which the staining of noninfected control cultures was set as 100%. Control cultures of noninfected GMK cell monolayers in the 24-well compartment confirmed that no virus spread occurred from the insert during the observation period.

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